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Characterization of Partially Purified Polyphenol Oxidase from Rhizome of Turmeric (*Curcuma longa* L.)

Ilesanmi, O.S.[1*], Olagunju, V.A[1,2] and Kayode, A.B.[3]

[1]Department of Biochemistry (Chemical Sciences), Achievers University, Owo, Ondo State, Nigeria.

[2]Department of Biochemistry and Chemistry, Miami University, Ohio, USA

[3]Department of Fruit and Spices Research, National Horticultural Research Institute (NIHORT), Ibadan, Nigeria.

Corresponding author e-mail: osilesanmi@achievers.edu.ng

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Abstract

Turmeric, a common spice with nutritional and nutraceutical applications undergoes brown reactions during harvest or post-harvest operations. This could possibly be due to presence of polyphenol oxidase (PPO) in the plant. In this work, isolation, partial purification and some characterization of PPO from rhizome of turmeric were described. The crude enzyme was purified using aqueous two phase partitioning. The partially purified PPO from turmeric was thereafter characterized. Aqueous two phase partitioning gave percentage yield and a final purification fold of 41% and 4.0 respectively. The optimum pH and temperature for the purified enzyme were 9.0 and 50 ^{o}C respectively. The enzyme was activated in the presence of some water-miscible organic solvents (DMSO>acetone>methanol). In contrast, the enzyme was inactivated in the presence of chloroform, butanol, petroleum ether and ethanol. In conclusion, the enzyme may find uses in several industrial and biotechnological applications.

Keywords: Polyphenol oxidase; Polyphenol; Aqueous two-phase partitioning; Turmeric; Biotechnological applications

1.0 Introduction

Polyphenol Oxidases (PPO) (E.C.1.10.3.1), also termed catechol oxidases, catecholases, diphenol oxidases, ortho-diphenolases, phenolases, and tyrosinases (Mayer, 2006). They are a group of copper-containing enzymes that catalyse the *o*-hydroxylation of monophenols to *o*-diphenols as well as the oxidation of *o*-diphenols to quinones in the presence of oxygen (Mirazizi *et al*., 2018; Qui *et al*., 2023). Polyphenol oxidase is broadly distributed among animals, fungi, and plants, although the studies are more extensive in plants (Moon *et al*., 2020; Ilesanmi *et al*., 2022). The study of PPOs in plants has focused primarily on their role in the process of postharvest browning, whereby cut or damaged plant tissues turn brown due to the polymerization of PPO-generated quinones, generating phyto-melanins (Mesquita and Queiroz, 2013). The enzyme can catalyze the oxidation of polyphenols and result in the browning of damaged or cut plant, which seriously affects quality and reduce the market value of the crop (Ilesanmi *et al*., 2021a; Qui *et al*., 2023). Polyphenol oxidase are widely present

in plants, play an important role in the growth, development, and stress responses. Many studies have reported that PPO is induced in response to biotic and abiotic stress in plants, and it has been implicated in several functional processes such as participating in plant defense and the synthesis of plant-specific metabolites (Sullivan and Hatfield, 2006; Araji *et al*., 2014).

Plant PPO generally contain three conserved regions, N-terminal cTP, aCuA and CuB domain and a C-terminus (Tran *et al*., 2012), which are responsible for thylakoid lumen localization and enzyme activity. Polyphenol oxidase are found in many plant species such as banana (Gooding *et al*., 2001), apple (Guardo *et al*., 2013), potato (Chi *et al*., 2014), eggplant (Jukanti and Bhatt, 2015), strawberry (Jia *et al*., 2016), red cocoyam (Ilesanmi *et al*., 2021b), bitter leaf (Ilesanmi *et al*., 2023a) etc. The function and distribution of PPO differ in various plants (Tran *et al*., 2012). Most PPO are transported to the thylakoid membrane in the chloroplast, or in cytosol and other organelles (Nakayamaa *et al*., 2001), whereas the phenolic compounds are localized to the vacuoles. Because of the different localization of the enzyme and its substrates, their interaction requires destruction of the cell and mechanical damage (Maioli *et al*., 2020). Plant PPOs have reported to possess various applications such as synthesis of drugs and other organic compounds (Ilesanmi *et al*., 2023b).

Turmeric (*Curcuma longa* L.) is a perennial rhizomatous crop of the Zingiberaceae, a world-wide known spice whose medicinal properties has received interest from both the medical and scientific world as well as culinary enthusiasts, as it is the major source of the polyphenol curcumin (Hewlings and Kalman, 2017). Turmeric contains 3 to 6% polyphenolic compounds which is known as curcuminoids (Abhishek and Dhan, 2008). It aids in the management of oxidative and inflammatory conditions, metabolic syndrome, arthritis, anxiety, and hyperlipidaemia. Most of these benefits can be attributed to its antioxidant and anti-inflammatory effects (Hewlings and Kalman, 2017). Like several plants, it suffers a reduction in its sensory qualities and health benefits as a result of spoilage which occurs due to browning. Recent research of novel anti-PPO systems is focused on mild alternatives to conventional treatments which could impair not only the sensory and nutritional properties of agro-food products but also the consumer health (Misra *et al*., 2017). Milder processes of controlling enzymatic browning can be discovered as PPO studies are being carried out and this will help improve the shelf-life of turmeric and its products.

Considering the vast economic importance of turmeric and wide applications of PPO, sufficient information on its relative occurrence and physio-chemical properties of the enzyme from turmeric is lacking and greatly necessary. Therefore, this work was carried out to establish the presence of PPO in the rhizome of turmeric, partially purify the enzyme, and investigate some biochemical properties of the purified *C. longa* PPO which could be exploited in the control of browning in turmeric and various biotechnological applications.

2.0 Materials and Methods

2.1 Materials

The fresh turmeric (*Curcuma longa*) was obtained from farms in Owo environs, Southwestern Nigeria. The plant was authenticated at the Department of Plant Science and Biotechnology, Achiever University, Owo, Ondo State, Nigeria.

2.1.1 Chemicals

3,4-dihydroxyphenyl-L-alanine (L-DOPA), catechol, L-tyrosine, pyrogallol, blue dextran, acetic acid, citric acid, sodium citrate, bovine serum albumin (BSA), PEG6000, ammonium sulphate, sodium phosphate dibasic (Na_2HPO_4), NaOH, anhydrous sodium phosphate monobasic ($NaHPO_4$), Coomassie brilliant blue R-250, glutathione was obtained from Sigma Chemical Company, St Louis, USA. All other reagents were of analytical grade.

2.2 Methods

2.2.1 Extraction of PPO from Turmeric

The fresh rhizome of turmeric (*Curcuma longa*) was cut, peeled and rinsed in distilled water. The cut plant was homogenized in 50mM potassium phosphate buffer, pH 6.5 on ice to obtain 30% homogenate. The homogenate was centrifuged at 10,000×*g* for 30 min at 4 ˚C using cold centrifuge in order to obtain clear crude supernatants. The supernatants were assayed for PPO activity using pyrogallol as substrate in a spectrophotometer at 410nm. The collected supernatant was stored in a freezer (at -20 ˚C) (Ilesanmi *et al*., 2014).

2.2.2 Standard Assay for Polyphenol Oxidase

Polyphenol oxidase activity was determined using L-Tyrosine, L-DOPA, catechol and pyrogallol as substrates in the crude supernatants and during purification according to the method of Wititsuwannakul *et al*. (2002) as modified by Ilesanmi *et al*. (2014). The reaction involved final concentration of 5 mM of the substrates, 50 mM phosphate buffer, pH 6.5 and appropriate volume of enzyme. Initial rate of product formation will be monitored spectrophotometrically.

2.2.3. Protein Concentration Determination

The protein concentrations in the crude homogenates and purified PPO was determined as described by Bradford in (1976) using BSA as the standard protein. The absorbance was read at 595nm in the spectrophotometer.

2.2.4 Purification of the Crude Extract

2.2.4.1 Aqueous Two-Phase Partitioning (ATPS)

The crude PPO was subjected to ATPS in accordance with the method of Srinivas *et al*. (1999) as modified by Ilesanmi and Adewale (2020). The polymers comprised of the mixture of PEG6000, ammonium sulphate and sodium chloride at 24, 7 and 2% w/v respectively. The polymer were continuously stirred with appropriate amount of enzyme until dissolved at 4 ^{o}C. The mixture was left on ice for 6 hr in the refrigerator. The mixture phase-separated thereafter into two layers (bottom and top layers). Both the bottom and top layer were assayed for PPO activity. Protein determination was also carried out. To remove salts from the bottom layer, it was dialyzed against 50 mM phosphate buffer, pH 6.5.

2.2.5 Characterization of the Partially Purified PPO from Turmeric

2.2.5.1 Substrate Specificity

Substrate specificity was carried out following the method of Wititsuwannakul *et al.* (2002) using: Monophenolic substrate - L-tyrosine, phenol; Diphenolic substrates – Recorcinol, Catechol, Methyl catechol, L-DOPA, Caffeic acid, 2-naphthol and Triphenolic substrates - Pyrogallol. The activity was monitored spectrophotometrically by oxidation of 5 mM substrates - L-DOPA at 475nm; Catechol at 410nm; L-tyrosine at 472nm; and pyrogallol at 510nm in 50 mM phosphate buffer, pH 6.5. The relative activities of the purified PPO for all the substrates were then compared.

2.2.5.2 Effect of pH on the Activity of *C. longa* PPO

The effect of pH on the PPO activity was determined using the pH range of 3.0 – 12.0 at room temperature. The following buffer systems at the indicated pH ranges was used: 50 mM citrate buffer, pH 3.0 - 4.0; 50mM Acetate Buffer, pH 4.5 – 6.0; 50 mM phosphate buffer, pH 6.5 – 8.0 and 50-mM glycine-NaOH buffer, pH 8.5 – 12.0, according to the method described by Sikora *et al*., (2019), The optimum pH for the PPO will then be determined using 0.05 M of pyrogallol as a substrate. PPO activity was calculated in the form of residual PPO activity at the optimum pH. The pH value corresponding to the highest enzyme activity was taken as the optimal pH.

2.2.5.3 Effect of Temperature on the Activity of *C. longa* PPO

The effect of temperature on purified PPO activity was investigated using the method as described by Sikora *et al*. (2019). This was carried out by incubating the reaction mixtures containing 50 mM phosphate buffer, pH 6.5- and 5-mM pyrogallol at temperatures ranging from 20 to 80 °C for 5 min after which the enzyme will be introduced and the activities observed plotted. The

residual PPO activity was then plotted against the different temperatures.

2.2.5.4 Effect of Organic Solvents on Purified *C. longa* PPO

The Effect of water-miscible (ethanol, methanol, acetone, butanol, DMSO) and water-immiscible (chloroform, petroleum ether) organic solvents on the activity of PPO was investigated as done by Harir *et al*. (2018). Organic solvents were introduced into the reaction mixture to achieve 0-70% final concentration. The residual activities were measured under standard assay conditions. The residual activity of the mixture without organic solvents was taken as 100%.

3.0 Results

3.1 Extraction and Purification

After the ATPS, the upper layer contained undesired proteins, unreacted polymers and other contaminants whereas the bottom layer was enzyme-rich. The partial purification of the *C. longa* PPO was summarized in Table 3.1. The percentage yield and purification fold obtained were 41% and 4.4 respectively.

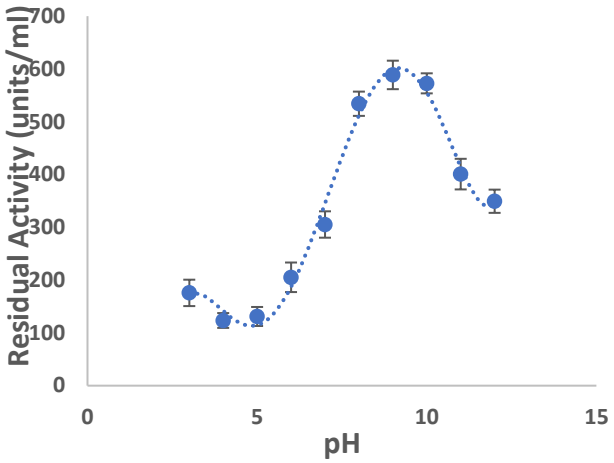

Figure 3.1: Effect of pH on the activity of partially purified PPO from *C.longa*. The PPO was introduced into different buffer system with varying pH and the PPO activity at the different pH were determined. The optimum pH obtained was 9.0.

Table 3.1: Purification Summary of Partially Purified *C. longa* PPO

Sample	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	Yield (%)	Purification Fold
Crude PPO	50.00	380.00	1.36	19,000	67.975	279.51	100	1.00
Purified PPO (ATPS)	12.60	593.00	0.48	7,709	15.901	1235.40	41	4.40

3.2 Substrate Specificity of Partially Purified PPO from *C. longa*

The substrate specificity of the purified PPO was carried out using mono-phenolic, di-phenolic and tri-phenolic substrates (Table 3.2). The percentage substrate specificity for pyrogallol, Catechol and L-DOPA were 100, 16 and 11% respectively. The highest activity was obtained in pyrogallol (a tri-phenolic substrate) which depicts it as the preferred substrate for *C. longa* PPO.

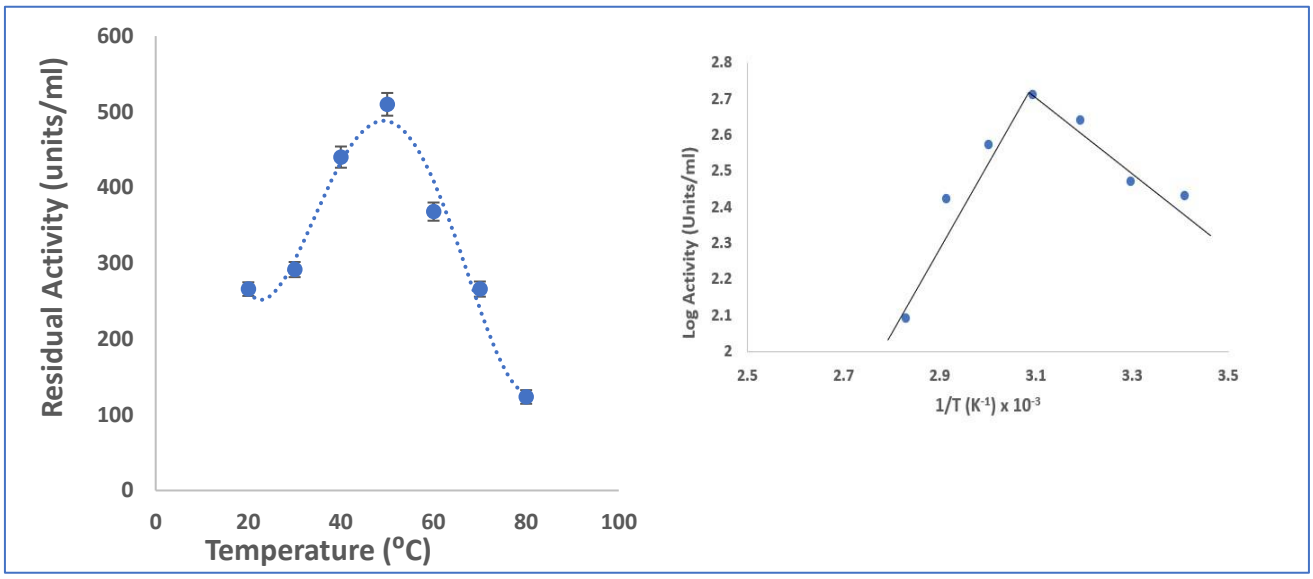

Figure 3.2: Effect of Temperature on the Activity of Purified PPO from *C. longa*. The PPO activity was determined at each temperature ranging from 20 to 80 ºC under the standard reaction conditions. The activities of the PPO were plotted against their respective temperature. The optimum temperature obtained was 50 ºC. Inset: Arrhenius plot for estimation of activation energy. The log of activity was plotted against inverse time in kelvin. The Estimated activation energy is 7.95J/mol.

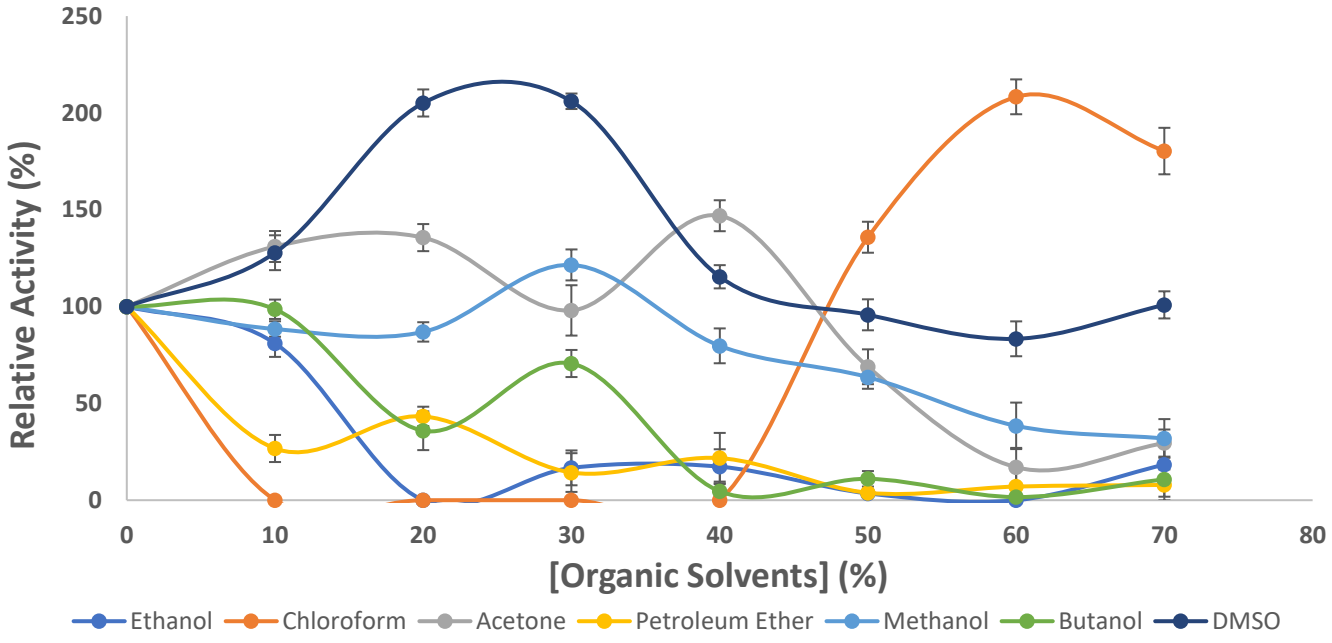

Figure 3.3: Effect of Organic Solvents on the Activity of Purified PPO from *C. longa*. The plot of % residual activities against different concentration of water-misicible and water-immisicible organic solvents. The activity of the purified PPO in 50 mM phosphate buffer was used as hundred percent (100%) activity.

3.3 Effect of pH on PPO from *C. longa*

The activities of PPO were plotted against pH (Figure 3.1). The highest activities were observed at alkaline pH range of between 8.0 and 10.0. The activity of the partially purified enzyme was optimum at pH 9.0. The enzyme was not active at acidic pH.

3.4 Effect of Temperature on PPO from *C. longa*

The effect of Temperature on PPO activity was carried out by incubating the reaction mixture containing the substrate and buffer at different temperatures. The optimum temperature was determined to be at 50°C (Figure 3.2). There was gradual decline in the activity at temperatures below or above the optimum temperature.

Table 3.2: Substrate Specificity of Purified PPO from *C. longa*

Substrates (%)	Relative Activity
Triphenols	
Pyrogallol	100 ± 0.0
Diphenols	
Catechol	16 ± 2.0
Methyl Catechol	0.0 ± 0.0
Caffeic Acid	0.0 ± 0.0
L-DOPA	11.0 ± 1.0
Resorcinol	0.0 ± 0.0
Monophenols	
L-tyrosine	0.0 ± 0.0
Phenol	0.0 ± 0.0
2-Naphthol	0.0 ± 0.0

3.5 Effects of Organic Solvents on PPO from *C. longa*

The summary of the effect of organic solvent is as shown in figure 3.3. The polyphenol oxidase from turmeric was inactivated gradually and lost activity almost completely in the presence of 20% (v/v) or above for butanol, ethanol, chloroform and petroleum ether. In contrast, the enzyme retained maximum activity in the presence of acetone, and methanol even at concentration up to 50% (v/v). interestingly, the enzyme maintained 100% activity at 70% (v/v) DMSO.

4.0 Discussion

Polyphenol oxidase has continued to be subject of intense investigation because of its versatility in several industrial and biotechnological processes. These enormous application of polyphenol oxidase necessitated further search for cheaper and readily available sources of the enzyme with better physico-chemical properties that could be deployed for new applications. In this work, the presence of PPO in the rhizome of turmeric (*C. longa*) was established. The crude PPO was subjected to non-conventional purification-aqueous two-phase partitioning (ATPS). The method was fast, less laborius and cost-effective, combining both purification and concentration of the resulting enzyme (Srinivas *et al*., 1999; Ilesanmi and Adewale, 2020; Ilesanmi and Adedugbe, 2023). The purification scheme proved efficient enough to give percentage yield and purification fold of 41% and 4 respectively.

Phenolics are crucial substrates of PPO (Peng *et al*., 2019) thus, the substrate specificity for *C.longa* PPO was determined using Monophenolic, Diphenolic and Triphenolic substrates. Highest *C. longa* PPO activity was obtained from pyrogallol, a triphenolic substrate and followed by catechol and L-DOPA, both which are diphenolic substrates. The enzyme had no activity in the presence of L-Tyrosine (monophenol). Thus, it can be concluded that *C. longa* PPO had preference for triphenolic and diphenolic substrates which support findings from previous studies that enzymes can have varying specificities for different substates depending on the source and cultivation conditions (Jukanti, 2017). Plants PPOs have different enzymatic activity on various phenolic substrates. The position and number of hydroxyl groups have remarkable effect on the PPO's activity. In addition, the specificity of polyphenol oxidase towards substrates are also influenced by the plant cultivar (Ilesanmi *et al*., 2023a).

Enzymes are considered extremely sensitive to pH as it affects surface charge, its solubility,

conformation and binding-ability with different substrates or inhibitors hence, an appropriate pH is important to obtain maximum activity (Ilesanmi *et al*., 2023a). The activity of *C. longa* PPO was carried out at varying pH of between pH 3.0 to 12.0. The optimum pH was obtained at pH 9.0. The optimum pH of *C. longa* PPO was different from green olive PPO (pH 4.5) using pyrogallol as substrate (Ben-shamol *et al*., 1977). Ilesanmi *et al*. (2023a) also reported optimum pH of 5.5 for PPO from bitter leaf. However, generally, plant PPO are reported to have pH ranging from 5.0 to 8.0 depending on the source of PPO, the extraction method, substrate and enzyme location (Teng *et al*., 2017).

Turmeric (*C. longa*) PPO showed an optimum temperature at 50 ℃ when the activity was plotted against their respective temperature ranging from 20 to 80 ℃. It was observed that the activity gradually increased from 20 to 50 ℃ and began to decline rapidly from 60 ℃ as it is postulated that the high temperature leads to denaturation of the enzyme and a resultant decrease in the enzyme activity (Zhang *et al.,* 2021). The finding was similar to optimum temperature for PPO by several researchers (Guven *et al.,* 2017).

Catalysis in organic solvent has been in vogue especially in the area of biotechnology and biocatalysis because when enzymes are placed in organic solvents, they tend to exhibit high selectivity and specificity, increased solubility of substrates and possibility of reduced microbial contamination (Ilesanmi and Adewale, 2020). The polyphenol oxidase from turmeric was inactivated gradually and lost activity almost completely in the presence of 20% (v/v) or above for butanol, ethanol, chloroform and petroleum ether. In contrast, the enzyme retained maximum activity in the presence of acetone, and methanol even at concentration up to 50% (v/v). interestingly, the enzyme maintained 100% activity at 70% (v/v) DMSO. It appeared the enzyme was discriminatory to water immiscible organic solvents. This further affirms *C. longa* PPO as a good industrial enzyme considering how DMSO is a common solvent used in many industries for various activities.

Conclusion

The presence of PPO in the rhizome of turmeric (*Curcuma longa*) has been established in this study. Partial purification method (ATPS) adopted was effective in removing unwanted proteins and contaminants from the desired enzyme. The enzyme was stable at 50 ^{o}C. The activity of the partially purified enzyme in the presence of DMSO, acetone and methanol could be exploited in several biotechnological applications.

CRediT authorship contribution statement

All authors contributed to Conceptualization, Methodology, Formal analysis, Investigation, Writing, and Visualization

Declaration of competing interest

The authors declare no known competing interests that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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